INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Bell & Howell Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI®
A STUDY OF THE EFFECTS OF GRAVITY ON THE GROWTH PATTERNS OF *ARABIDOPSIS* ROOTS USING IMAGE ANALYSIS TECHNIQUES

DISSEMINATION

Presented in Partial Fulfillment of the Requirements for

The Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Jack L. Mullen, B.S.

* * * * *

The Ohio State University
2000

Dissertation Committee:

Dr. Elizabeth Gross, Advisor
Dr. Michael L. Evans
Dr. Hideo Ishikawa
Dr. Richard Sayre

Approved by
Advisor
Biophysics Program
ABSTRACT

Although Arabidopsis is an important system for studying root physiology, the localized growth patterns of its roots have not been well defined, particularly during tropic responses. We have developed an automated video digitizer system with custom image analysis software to investigate the gravitropic response of Arabidopsis thaliana (L.) Heynh roots with high resolution.

By applying small charcoal particles to the root surface and analyzing their displacement during growth, we were able to characterize growth rate profiles along the apex of the primary root. When growing vertically, the elongation zone extended back to approximately 900 µm from the tip. The distal elongation zone (DEZ) has previously been described as the apical region of the elongation zone in which the relative elemental growth rate (REGR) is ≤ 30% of the peak rate in the central elongation zone (CEZ). By this definition, our data indicate that the basal limit of the DEZ was located 250 µm from the tip. However, within the first hour after gravistimulation, the growth patterns of the root changed. The basal limit of the DEZ shifted acropetally on the upper side of the root, due to a combination of increased growth in the DEZ and growth inhibition in the CEZ. On the lower side, the DEZ shifted basipetally as the REGR decreased. When roots were treated with the auxin-transport inhibitor, NPA, they became agravitropic.
However, while growth in the CEZ was inhibited, the growth pattern in the DEZ was unaffected.

We also studied the kinetics of the gravitropic response of the mutant *rgrl* (reduced gravitropism in roots) to investigate the interactions of gravity and other environmental stimuli. Although *rgrl* had a smaller rate of curvature than wild type, curvature was initiated in the same region of the root, the DEZ. However, the gravitropic response of *rgrl* contained a feature not found in the wild type: there was often an upward curvature that initiated in the CEZ. Because this response was dependent on the tactile environment of the root, it most likely resulted from the superposition of the waving/coiling phenomenon onto the gravitropic response. We found that the frequencies of the waving pattern and circumnutation (a cyclic endogenous growth pattern) were similar, so the waving/coiling phenomenon is likely governed by circumnutation patterns.

By connecting a rotating stage platform to the digitizer system through a feedback mechanism, we were also able to maintain a constant angle of gravistimulation to *Arabidopsis* roots for long time periods. Using this technique we were able to avoid the problem that as a root responds, the gravitational stimulus normally changes. The rate of curvature for a constant stimulus approximated the sine rule for angles between 20° and 120°. For a given angle of stimulation, the rate of curvature remained constant, with no observed attenuation of the response. While previous reports of *Arabidopsis* root gravitropism suggest latent periods of 30 min., using a smooth mechanical stage to reorient the root, we observed a mean time lag of approximately 10 min. This more rapid onset can, in part, be explained by reduced mechanical perturbation during the process of
gravistimulation. This suggests that mechanical stimulation associated with rapid root reorientation may confound investigations of early gravitropic events.
ACKNOWLEDGMENTS

I wish to thank Michael L. Evans and Hideo Ishikawa for their guidance. I would also like to thank my committee members, Elizabeth Gross and Richard Sayre for their time and interest in this project. I am grateful to Chris Wolverton for the daily discussions and light-hearted atmosphere. This research was supported by the National Science Foundation, the National Aeronautics and Space Administration, and the National Aeronautics and Space Administration/National Science Foundation Joint Program in Plant Biology, Network for Research on Plant Sensory Systems.
VITA

May 30, 1971 ...........................................Born – Warren, Ohio

1993 .....................................................B.S. Physics, University of Dayton

1993 - 1994 ..........................................University Fellowship, The Ohio State University

1994 – present .................................Graduate Teaching and Research Associate,
                                    The Ohio State University

PUBLICATIONS

Research Publication

growth rate patterns in the elongation zone of Arabidopsis roots upon
gravistimulation. Planta 206: 598-603

ML (1998) Root-growth behavior of the Arabidopsis mutant rgr1: roles of
gravitropism and circumnutation in the waving/coiling phenomenon. Plant Physiol
118: 1139-1145

by an applied electric field. J Plant Res 112: 493-496

gravitropic stimulus responses in Arabidopsis roots using a feedback system. Plant
Physiol, in press.

FIELDS OF STUDY

Major Field: Biophysics
TABLE OF CONTENTS

Abstract .................................................................................................................................. ii
Acknowledgments ................................................................................................................. v
Vita ......................................................................................................................................... vi
List of Figures ....................................................................................................................... x
List of Tables ....................................................................................................................... xii
Chapters:
1. Introduction ....................................................................................................................... 1
2. Relative elemental growth rate patterns in the elongation zone of Arabidopsis roots .... 10
   2.1 Materials and methods ................................................................................................. 12
       2.1.1 Plant materials and growth conditions .............................................................. 13
       2.1.2 Fixation and sectioning ...................................................................................... 13
       2.1.3 Determination of growth rate distribution for vertical roots ...................... 13
       2.1.4 Determination of growth rate distributions in gravistimulated roots .......... 16
       2.1.5 Determination of transverse variations in marker motion ......................... 17
2.2 Results ............................................................................................................................ 17
       2.2.1 Vertical elongation ............................................................................................. 17
       2.2.2 Post-gravistimulation elongation ....................................................................... 18
2.2.3 Elongation of roots treated with an auxin-transport inhibitor ............................................................... 24

2.3 Discussion ..................................................................................................................................................... 24

3. Root-growth behavior of the Arabidopsis mutant rgr1: roles of gravitropism and circumnutation in the waving/coiling phenomenon .......... 30

3.1 Materials and methods ............................................................................................................................... 33

3.1.1 Plant materials and growth conditions ................................................................................................. 33

3.1.2 Video-digitizer system .......................................................................................................................... 34

3.1.3 Interaction of gravitropism and tactile stimulation ........................................................................... 35

3.1.4 Circumnutation and calculation of curvature .................................................................................... 37

3.1.5 Conventions of terminology ............................................................................................................ 37

3.2 Results ....................................................................................................................................................... 38

3.2.1 Gravitropism and the waving response ............................................................................................. 38

3.2.2 Upward curvature and altered tactile and light environments ....................................................... 43

3.2.3 Circumnutation .................................................................................................................................. 45

3.3 Discussion .................................................................................................................................................. 48

4. Kinetics of constant stimulus responses in Arabidopsis roots using a feedback system ................................................................. 53

4.1 Materials and methods ............................................................................................................................... 54

4.1.1 Plant materials and growth conditions ............................................................................................. 54

4.1.2 The feedback system .......................................................................................................................... 55

4.2 Results ....................................................................................................................................................... 56

4.2.1 Gravitropism kinetics .......................................................................................................................... 56

4.2.2 Dependence on stimulation angle ....................................................................................................... 59
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Changes with development in the orientation of a root vis-à-vis gravity</td>
<td>3</td>
</tr>
<tr>
<td>1.2</td>
<td>DIC micrograph of a root tip of <em>Arabidopsis</em></td>
<td>3</td>
</tr>
<tr>
<td>2.1</td>
<td>Application of charcoal markers along the axis of an <em>Arabidopsis</em> root</td>
<td>14</td>
</tr>
<tr>
<td>2.2</td>
<td>Velocity field for vertically growing <em>Arabidopsis</em> roots</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Relative elemental growth rate profile for a vertically growing <em>Arabidopsis</em> root</td>
<td>19</td>
</tr>
<tr>
<td>2.4</td>
<td>Measurement of transverse variations in growth</td>
<td>20</td>
</tr>
<tr>
<td>2.5</td>
<td>Comparison of REGR profiles for the upper and lower flanks of a representative <em>Arabidopsis</em> root 45 min after gravistimulation</td>
<td>22</td>
</tr>
<tr>
<td>2.6</td>
<td>Growth pattern for <em>Arabidopsis</em> roots treated with 10 μM NPA</td>
<td>25</td>
</tr>
<tr>
<td>3.1</td>
<td>Waving/coiling patterns for seedlings of Ws and <em>rgrl</em></td>
<td>31</td>
</tr>
<tr>
<td>3.2</td>
<td>Logic of custom software for measurement of length and orientation of roots</td>
<td>36</td>
</tr>
<tr>
<td>3.3</td>
<td>Kinetics of the gravitropic response of a representative wild-type root growing along the surface of a vertically oriented agar plate</td>
<td>39</td>
</tr>
<tr>
<td>3.4</td>
<td>Kinetics of the gravitropic response of a representative root of an <em>rgrl</em> seedling growing along the surface of a vertically oriented agar plate</td>
<td>42</td>
</tr>
<tr>
<td>3.5</td>
<td>Kinetics of the gravitropic response of a representative root of <em>rgrl</em> growing through a block of agar</td>
<td>44</td>
</tr>
<tr>
<td>3.6</td>
<td>Circumnutation patterns for roots growing through a block of agar</td>
<td>46</td>
</tr>
</tbody>
</table>
3.7 Waving growth pattern for roots growing along the surface of an agar plate .................................................................47

3.8 A cross-sectional representation of changes in the root tip orientation of a typical wild-type seedling .........................................................49

4.1 Feedback system for constraining root orientation .........................57

4.2 Kinetics of the gravitropic response of an Arabidopsis root ...........58

4.3 Early time-course for the gravitropic response of typical Arabidopsis roots .................................................................60

4.4 The dependence of the rate of root curvatures on the stimulation angle …61

4.5 Threshold of gravitropic response in Arabidopsis roots ..................63

4.6 Localized changes in Arabidopsis root orientation upon gravistimulation .................................................................64

4.7 Relative local contributions to gravitropic curvature ......................66
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Comparison of growth profile features for vertical and gravistimulated <em>Arabidopsis</em> roots</td>
<td>23</td>
</tr>
<tr>
<td>3.1</td>
<td>Comparative gravitropism kinetics for roots of wild-type and <em>rgr1</em> Seedlings growing on the surface of vertically oriented agar plates</td>
<td>41</td>
</tr>
<tr>
<td>A.1</td>
<td>Accuracy of root orientation measurements</td>
<td>74</td>
</tr>
<tr>
<td>A.2</td>
<td>Accuracy of root tip position calculations</td>
<td>74</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

Gravity has provided a nearly constant directional force throughout the evolution of life. Gravity, therefore, provides a reliable indicator for a plant of its surroundings. Plants have developed mechanisms to utilize this indicator so that the shoot of a seedling grows antiparallel to the gravity vector, allowing the leaves to reach sunlight. This is a directional growth response to the directional stimulus of gravity (gravitropism).

Likewise, by guiding roots downward, the plant’s response to gravity reduces the likelihood of a root growing into the air and drying out. Gravity’s influence on plant growth continues throughout the life of the plant and plays a large role in the overall form of the plant, and this effect of form can translate into a significant impact on fitness and productivity. For example, gravity is thought to influence reaction wood formation (Hejnowicz 1997), which is important for supporting shoot mass for photosynthesis.

Photosynthetic efficiency is also influenced by the orientations of branches and leaves, which are determined, in part, by gravity. In white light, for instance, Arabidopsis rosette leaves are oriented horizontally. But if placed in the dark, the leaves raise to a more vertical orientation (Hangarter 1997). Since both light and gravity have dramatic effects on plant form, the ability of a plant to integrate both environmental cues into an overall
response is also an area of importance. Although the perception systems for gravitropism and phototropism are different, the idea that these systems share downstream functions is supported by the identification of mutations which alter both responses (Liscum and Briggs 1996). The red light dependence of gravitropism in some plants (Liscum and Hangarter 1993, Lu et al. 1996) also argues for an interaction between light and gravity responses. Yet up until this point detailed physiological studies of interactions between these signaling pathways have been limited by the lack of a well-characterized dose-response for gravitropism and by changes in the gravistimulus caused by phototropic curvature.

Arguably, the most studied gravity response in plants has been tropic curvatures in coleoptiles and roots. Early studies, primarily with coleoptiles, provided insights not only concerning environmental responses, but also for organ formation and for the discovery of phytohormones (see Went and Thimann 1937). Roots are an important system to study, as their function is vital throughout the life of a plant. The architecture of the root system is dependent on the interactions of many environmental cues. The responses to these environmental stimuli can change with the developmental state of the root system (Firn and Digby 1997, and this study Figure 1.1), providing a system for correlating developmental changes with alterations in the gravitropic response. Yet, the primary root of a seedling provides a geometrically simple system which facilitates the study of the response.

Research into plant gravitropism has been dominated by two concepts: the starch-statolith hypothesis and the Cholodny-Went hypothesis. The starch-statolith hypothesis was independently elaborated by Haberlandt and Němec in 1900 (see Haberlandt 1914)
**Figure 1.1.** Changes with development in the orientation of a root vis-à-vis gravity. Data are from a representative maize secondary root, showing a change to a steeper, though non-vertical, orientation upon emergence.

**Figure 1.2.** DIC micrograph of a root tip of *Arabidopsis*. Arrows indicate sedimented statoliths, which are concentrated in the columella cells of the root cap. Photograph courtesy of C. Wolverton.
to explain how a plant could perceive gravity and was based on the idea that gravity could cause the sinking of the heavier bodies within a cell. They proposed that the starch grains which in some cells sediment under the influence of gravity (statoliths, Figure 1.2) provide information about the orientation by interacting with some component of the bottom of the cell. Correlative evidence that these starch grains are necessary for gravitropic response was provided by the findings that the root cap, which contains the statoliths, is necessary for gravitropic response (Darwin 1880, Hillman and Wilkins 1982) and that degrading starch by hormonal treatments affects gravitropic sensitivity (Volkmann and Sievers 1979). More recent support for the starch-statolith hypothesis came from studies in which statolith-containing cells in the root cap were ablated with a laser, resulting in a reduced response (Blancaflor et al. 1998). And studies with starch-deficient mutants of *Arabidopsis* revealed a correlation between starch content and gravitropic sensitivity (Kiss et al. 1996).

While it appears that the starch grains in the root cap do indeed confer gravitropic sensitivity, it is unclear with what cellular components these statoliths interact in order to provide directional information. As originally envisioned the statoliths sedimented to the bottom of the cell where they interacted with the cell membrane. The resulting statolith-membrane contact was postulated to block plasmadesmata (Juniper 1976) or release bound hormone (Kaufman et al. 1987). Studies attempted to give the starch-statolith hypothesis a more quantitative basis by examining the relationship between statolith-membrane contact and the magnitude of the gravitropic response (Audus 1964, Perbal 1974). These investigators found that the magnitude of the response depended sinusoidally on the angle to which the root was rotated, and they correlated this with the
number of statoliths reaching the bottom of the cell. Due to steric hindrances and apparent friction between statoliths, the greatest statolith-membrane contact occurred at angles greater than 90°, which agreed well with their observed optimal response at 120°-135°. However, in roots, starch grains may never reach the plasma membrane (Moore and Evans 1986), and the sinusoidal relationship between the response and stimulation angles has also been called into question (Barlow et al. 1993). Hence, the details of perception of gravity remain obscure.

While the mechanism by which the plant perceives gravity is unclear, the stimulus soon causes a difference in growth between the upper and lower flanks of the root, resulting in curvature of the organ. The Cholodny-Went hypothesis sought to unite tropic curvature with the concept of hormonal control of growth to form a simple model of growth regulation. The Cholodny-Went hypothesis states that growth curvatures are due to an unequal distribution of auxin between the two sides of the curving organ, which is brought about by lateral transport of auxin in response to the stimulus (Went and Thimann 1937). This hypothesis stemmed from a series of experiments, originally in coleoptiles and then extended to roots, which found that the tip of the organ produces auxin, the hormone which controls growth of the organ. Asymmetrical application of auxin induced curvature, and the application of unilateral light led to an asymmetrical distribution of auxin in the tip, which was postulated to move basipetally to the growing region. Polar transport of auxin has also been found following gravistimulation (Harrison and Pickard 1989, Young et al. 1990). Genetic approaches have also begun to identify putative auxin transport proteins (Bennett et al. 1996, Luschnig et al. 1998). Mutations in these genes result in altered gravitropic response. Hence, auxin redistribution seems to
be involved in gravitropism at some level. Part of the initial evidence in favor of the Cholodny-Went hypothesis was the finding that following gravistimulation, neither the total amount of auxin nor the growth rate of the organ changed (Navez 1933, Navez and Robinson 1933, Dolk 1936, Went and Thimann 1937). These researchers found only a movement of auxin so that there was more auxin in the bottom half of the organ than there was on the top. Likewise, they found that the growth rate on the top increased in proportion to the decrease on the lower side, with no net change in growth rate.

While the original Cholodny-Went hypothesis implied that the growth rate on the upper side of the root should increase and that the rate on the lower side should decrease, there are many patterns of differential growth, which lead to curvature. Increased study of root growth led to reports that growth on both the upper and lower sides of roots was inhibited, with the growth of the lower side inhibited to a greater extent (Rufelt 1957, Bennet-Clark et al. 1959, Bejaoui and Pilet 1977). This led to increased study of possible growth inhibitor production in the root cap. However, with improved experimental techniques, it was found that the initial curvature after gravistimulation in roots of Lepidium (Selker and Sievers 1987) and maize (Ishikawa et al. 1991) is caused by increased growth on the upper side and decreased growth on the lower side. However, the overall growth rate does change. These studies also revealed that the curvature is not constant along the elongating portion of the root. Further, the differential growth patterns change with time, often even reversing in direction. These differential growth patterns are more complex than predicted by the Cholodny-Went hypothesis. It has been suggested that temporal changes in differential growth could be caused by changes in auxin sensitivity (Evans 1991). While there is evidence that the sensitivity of cells to
auxin can be changed by adding exogenous auxin (Gougler and Evans 1981, Rorabaugh and Salisbury 1989), it remains unclear whether such alterations in auxin sensitivity can explain the details of the gravitropic response.

Studies on the location of the gravitropic response in the roots of cress (Selker and Sievers 1987) and maize (Ishikawa et al. 1991) found that curvature initiated in a region apical to the rapidly elongating region of the root. Gravistimulation was also found to induce rapid changes in the electrical properties of these cells in Vigna (Ishikawa and Evans 1990a) and maize (Collings et al. 1992). The asymmetry of these electrical changes between the upper and lower sides of the root suggests that they may be important in the transduction of the gravitropic response. Since these changes were not seen in the rapidly elongating region of the root, the results suggest that, in vertically growing roots, the region of rapid elongation, or central elongation zone (CEZ), is a physiologically distinct region from the more distal region of cells, which have not yet begun to rapidly elongate (distal elongation zone, DEZ). The cells in the DEZ also play an important role in the growth response to other environmental signals such as touch (Ishikawa and Evans 1992) and water stress (Sharp et al. 1988). Yet, while these cells are important in the response to gravity, as well as other stimuli, they present a difficulty of the Cholodny-Went hypothesis. The DEZ is able to initiate strong gravitropic curvature even if the root is pretreated with high levels of auxin, which severely inhibit overall root elongation (Ishikawa and Evans 1993, Muday and Haworth 1994). Since auxin pretreatment should cause the auxin levels to be high on both the upper and lower sides of the root, these results indicate that auxin might not control differential growth in the DEZ.
Because of the special status of the DEZ in the response to environmental signals, its growth patterns need to be studied in more detail. Most earlier studies of differential growth focused on the overall growth rates for the upper and lower flanks of the root (see Fim and Digby 1980). Yet to make sense of those growth rates, we need to understand the profile of growth along the root axis and the relationship of growth regulation in the DEZ and CEZ. The numerous mutants and transgenic lines available in Arabidopsis thaliana, the model system for plant genetics, offer potential for providing clues into the growth regulation mechanism. And the small size of Arabidopsis allows a response to be narrowed to a small population of cells. Yet despite the potential benefits using Arabidopsis, most studies of growth responses have utilized other species such as maize or cress, which have larger, less fragile roots. And even recent image analysis techniques with these larger roots have yielded results limited to very general DEZ-CEZ distinctions (Jaffe et al. 1985, Selker and Sievers 1987, Ishikawa et al. 1991). Magnified images of coleoptiles and roots have also been projected onto moving photographic film. And while this streak photography method (Erickson and Sax 1956) may have high resolution, it is limited in applicability to straight growth. Alternative methods of time-lapse photography analysis have been employed (Silk and Erickson 1978, Beemster and Baskin 1999), though the sampling frequency has been too low to be useful in quantifying the early time-dependent changes in gravitropic curvature. Also, since the data are only known after the fact, real-time interventions are not possible. We, therefore, wanted to develop an image-analysis system suitable for investigating Arabidopsis root growth, and particularly gravitropism, in detail. This system needed the magnification to accurately measure changes in growth over short time periods, the spatial resolution in the data...
analysis to compare responses in the DEZ and CEZ, and the temporal resolution to quantify the time-dependent response to a gravitropic stimulus. By obtaining detailed data on the growth of *Arabidopsis* roots and differences from the wild-type in several mutant and transgenic lines, we have gained a better understanding of plant root growth in general, and the response to environmental stimuli. This information will provide a framework in which to better evaluate models of plant growth regulation, such as the Cholodny-Went hypothesis, and allow us to refine the models to better explain growth behavior.
CHAPTER 2

RELATIVE ELEMENTAL GROWTH RATE PATTERNS IN THE ELONGATION ZONE OF *ARABIDOPSIS* ROOTS

Due to the availability of a variety of mutants with altered root behavior, *Arabidopsis* seedlings have become increasingly popular for studies of root physiology, including investigations of gravitropism (Hobbie and Estelle 1995, Simmons et al. 1995a, Ishikawa and Evans 1997), root slanting/coiling (Okada and Shimura 1990; Simmons et al. 1995b, Rutherford and Masson 1996), and auxin responsiveness (Maher and Martindale 1980, Evans et al. 1994). Unfortunately, the small size of *Arabidopsis* roots and their relatively slow growth rate make them less than ideal for detailed studies of stimulus-induced changes in localized growth rate patterns.

In spite of these difficulties, there is a need to better understand the growth behavior of localized regions of the *Arabidopsis* root and the extent to which different regions of the elongation zone contribute to root growth responses such as gravitropism and waving. Studies with the larger roots of maize have shown that there are differences in the physiology of cells in different regions of the elongation zone and that different cell populations within the elongation zone respond to environmental signals in different ways. Cells near the distal end of the elongation zone, for example, differ from cells in
the central part of the elongation zone with regard to auxin responsiveness, role in gravitropism, and a variety of other responses (Baluška et al. 1994, Ishikawa and Evans 1995). We have referred to the region of the root between the meristem and the central elongation zone (CEZ) as the distal elongation zone (DEZ) (Ishikawa and Evans 1995).

In the case of gravitropic response, small groups of cells in the DEZ have been implicated in the initiation of curvature (Zieschang and Sievers 1991, Ishikawa and Evans 1993).

The cells of the DEZ and CEZ show differences in their response to auxin, a hormone thought to play a role in establishing differential growth. These zones also differ in changes in electrical properties (Ishikawa and Evans 1990b), which manifest during the graviresponse. These observations suggest that different mechanisms may be involved in the responses of these two regions to environmental signals. Understanding the differences in auxin responsiveness and the changes in growth patterns during the graviresponse will help clarify how these physiological differences between the DEZ and CEZ translate into growth responses and allow us to delimit regions of the root based upon these responses. In the small primary root of Arabidopsis, such regions may be composed of only a small number of cells.

In plants with large roots, such as maize, the pattern of relative elemental growth rate (REGR) has been well-characterized (Erickson and Goddard 1951, Erickson and Sax 1956). More recently, Ishikawa and Evans (1993) described the importance of the DEZ in the gravitropic response of maize roots, providing a functional definition for the DEZ. However, due to the technical problems presented by Arabidopsis roots, attempts to map growth rate patterns in this model system have been limited. Estimates of the boundaries of the elongation zone for roots of the Arabidopsis thaliana ecotype Landsberg (Okada
and Shimura 1990) and the sku mutants (Rutherford and Masson 1996) have been made by placing graphite beads along the root surface and photographing them at 1-h intervals. These studies suggested that the elongation zone was located in the region between 250 μm and 750 μm from the root tip. In both of these studies, root growth data were collected over a period of at least 7 h. Because the roots were growing at 120—160 μm h⁻¹, the roots would have extended by at least 840 μm during the study, displacing most of the markers from the elongation zone. Recently the growth rate distributions along the root axis have been measured (Baskin et al. 1995, Beemster and Baskin 1998), providing a more detailed characterization of the elongation zone. However, the study by Baskin et al. (1995) used cell shape data to determine elongation rates, without measuring cell division. Beemster and Baskin (1998) addressed these inadequacies by directly measuring the velocity of applied markers, though the interval between observations was 1 h. During this time the root extended by about 20% of the elongation zone. To obtain measurements of the pattern of elongation in Arabidopsis roots with less temporal averaging, we used a computer-based video digitizer system that automatically tracks the location of marker beads on the root and stores their coordinates every 20—30 s. Using this system, we can analyze growth performance of the root over relatively short time periods (e.g. 20 min). This improved resolution allowed us to obtain a detailed description of the profile of elongation rates along the root apex and to analyze time-dependent changes in the REGR profiles that result from gravistimulation.

Materials and methods
Plant material and growth conditions. Seeds of *Arabidopsis thaliana* (L.) Heynh (ecotype Columbia) were surface-sterilized in 5.25% NaOCl, rinsed in sterile distilled water, and plated on 1% (w/v) agar containing half-strength Murashige-Skoog medium (Murashige and Skoog 1962), 1 mM Mes, and 1% (w/v) sucrose, final pH 5.8. For some experiments, a small volume of stock NPA (N-1-naphthylphthalamic acid) in DMSO was added to the molten agar for a final concentration of 10 μM. Plates were vernalized at 4°C for 1-4 d and then placed under continuous illumination from cool-white fluorescent lamps (model F30t8-CW; Sylvania, Danvers, Mass., USA; fluence rate approximately 50 μmol m⁻² s⁻¹). The seedlings were used after being grown at 23-25 °C for 4-6 d.

Fixation and sectioning. Vertically growing roots were fixed and sectioned in order to observe cell shape in the root elongation zone. Vertical roots were left overnight in a fixative of microtubule stabilizing buffer (50 mM PIPES, 5 mM MgSO₄, 5 mM EGTA) containing 3.7% (v/v) formaldehyde and 1.0% (v/v) glutaraldehyde. They were then rinsed in distilled water and dehydrated in a graded ethanol series. The roots were then embedded in Spurr's resin for sectioning. The 3-μm sections were stained with safranin-O for visualization of cell walls.

Determination of growth rate distribution for vertical roots. Marker beads made from ground charcoal (mean diameter = 33 μm) were applied along the axis of the root tip, as shown in Figure 2.1. We used a video digitizer system with custom software (based upon that of Ishikawa et al. 1991) to record the coordinates of the markers every 20-30 s. The resolution of the digitizer system was 1.85 μm by 2.45 μm. Images of the root were
**Figure 2.1.** Application of charcoal markers along the axis of an *Arabidopsis* root. The mean diameter of the markers is 33 μm. The white points on the markers indicate the calculated position of the marker. Bar = 50 μm
Figure 2.2. Velocity field for vertically growing *Arabidopsis* roots. The data points represent the velocities at which markers are moving away from the root tip at different distances from the tip. The *solid line* is the logistic model fit of the data.
saved every 10 min. Using the images to find the points along the boundaries of the root, a third-order polynomial was fitted to the axis of the root: this served as the axial spline. To find a marker’s distance from the root tip, we first projected the marker onto the axial spline. The position of this projection was the point on the spline whose normal intersected the center of the marker. The distance was then calculated by determining the arc length along the spline, \( f(x) \), from the root tip to the projection of the marker, given by:

\[
\int_{r_{tip}}^{r_{marker}} \sqrt{1 + f'(x)^2} \, dx
\]

The velocity of a marker was found by calculating the rate of change in the distance of a marker from a reference marker at the tip of the root. This was accomplished by a linear regression fit of marker distance versus time for a 20-min time interval. Since the shape of the root axis can change noticeably during this time interval, especially in the gravistimulated roots, we used two splines for the calculations: one representing the root at the beginning of the interval and the other representing the final orientation. We created a velocity field (Figure 2.2) from the time-weighted average of the data from the two splines, and we modeled this growth using the logistic equation described by Morris and Silk (1992). Differentiating the logistic equation with respect to position provided the REGR profiles.

**Determination of growth rate distributions in gravistimulated roots.** After the roots were gravistimulated by rotation to a horizontal orientation, separate growth profiles were determined for the upper and lower sides. Because practical considerations prevented us
from placing actual markers along the upper and lower surfaces, we created peripheral splines for the upper and lower edges using the same method as for the axial spline, except only using points along the upper or lower edge, respectively. The markers were then projected onto the peripheral splines. The velocities of the projections were then used to find the REGR profiles as described above.

**Determination of transverse variations in marker motion.** To see if growth patterns on the flanks of a gravistimulated root could be described using the movement of markers placed along the axis of the root, we measured the velocity of markers, relative to the tip, at different transverse positions. Pairs of markers were placed at approximately the same distance from the tip, but on opposite flanks of the root. At 10-min intervals we projected the markers onto the axial spline and calculated the distance between the projections of markers in a pair, as described above. For pairs of projections initially less than 15 µm apart, we then fitted the change in the distance between markers versus time by linear regression.

**Results**

**Vertical elongation.** Figure 2.2 shows a typical velocity field of marker beads at different axial positions along a vertically growing root. The velocity profile conforms to an asymmetrical logistic curve, as can be seen by the fit of the logistic model ($R^2 = 0.999$). Differentiating the logistic equation with respect to position yields the REGR as a
function of distance from the root tip. Figure 2.3 shows a representative REGR profile for a vertically growing root. By this analysis, the location of the peak REGR was 481 ± 50 μm (mean ± SD, n = 8) from the extreme root tip (tip of the root cap). In order to estimate how far the elongation zone extends from the tip of the root, we calculated the point along the root axis at which 95% of growth occurred distally. By this definition, the basal limit to the elongation zone was 912 ± 137 μm from the tip. Because this area of the root is in the asymptotic region of the velocity field, the standard deviation of these data is large. However, an estimate of approximately 900 μm from the tip agrees with our observations of slight, though detectable movement of markers in this region. We also wanted to determine the boundary between the DEZ and the CEZ. The boundary between these two regions was previously defined as the location along the root axis at which the REGR equals 30% of the peak REGR (Ishikawa and Evans 1993). Using this definition, we found the boundary between the DEZ and the CEZ to occur 248 ± 30 μm from the root tip.

Post-gravistimulation elongation. Having detailed the REGR profile of vertically growing roots, we were then able to investigate how the growth patterns changed in response to gravistimulation. However, in practice it was not possible to place a sufficient number of markers on both flanks of the root to provide a well-defined velocity field. Therefore, we tested whether a marker placed along the center of the root could accurately describe the growth patterns of the upper and lower sides of the root. As shown in Figure 2.4A, this was accomplished by first projecting the markers, which were placed near the root edges, onto the axis and calculating the axial distance between pairs
Figure 2.3. Relative elemental growth rate profile for a vertically growing *Arabidopsis* root. The basal limit of the DEZ is defined as that point in the apical region of the elongation zone where the REGR is 30% of the maximum REGR. The *inset* is a longitudinal section of the root and is scaled to the graph.
Figure 2.4. Measurement of transverse variations in growth. A. Method for determination of transverse variations in marker motion. The marker centers are projected onto the axial spline of a gravistimulated root. Changes in the difference in the position of these projections for a pair of markers on opposite flanks of the root are then determined as a function of time after gravistimulation. The inset shows an actual image of such a root (scale bar length is 50 μm). B. Mean values of the difference in position for markers in the CEZ (n = 9) or DEZ (n = 6). The dotted lines delimit the 95% confidence intervals for the fitted lines.
of markers. By calculating a linear regression fit of changes in this distance, we measured transverse variations in the velocity of movement away from the tip at a particular distance. During the first hour after gravistimulation, neither pairs of markers in the DEZ nor those in the CEZ showed variations in velocity which were significantly different from zero (Figure 2.4B). Differences in elongation rate between the two flanks of the root are accounted for by curvature of the root axis at the location of differential growth, without differential changes in axial velocity. Thus, markers placed along the center of the root can be used to accurately describe the growth patterns along the root flanks.

By projecting the markers to the edges of the root, we determined growth profiles for gravistimulated roots. The midpoint of the time interval used for the calculations was 45 min after gravistimulation. Figure 2.5 compares the vertical growth profile of a representative root with the REGR profiles for the upper and lower sides after gravistimulation. In the DEZ, the REGR increased on the upper flank of the root, while decreasing on the lower flank. If we define the DEZ-CEZ boundary based on the peak REGR for the vertical condition, then the boundary on the upper side shifted toward the root apex during the gravitropic response. Table 2.1 gives the mean locations of REGR features for the upper and lower sides of the stimulated roots in comparison to the vertical controls. For the upper side, the position of the DEZ-CEZ boundary was 183 ± 76 μm (mean ± SD, n = 6) from the root tip and represented a significant change from the vertical location (P < 0.05). Conversely, on the lower side, the basal limit of the DEZ shifted basipetally (P < 0.10) to a location 306 ± 51 μm from the tip. On the upper side, the position of the peak REGR also shifted apically (P = 0.10, see Table 2.1). However,
Figure 2.5. Comparison of REGR profiles for the upper and lower flanks of a representative *Arabidopsis* root 45 min after gravistimulation. The *dashed line* shows the vertical profile. The *inset* shows images of the root at the beginning and end of the observation period.
<table>
<thead>
<tr>
<th></th>
<th>Vertical</th>
<th>Gravistimulated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Upper side</td>
<td>Lower side</td>
</tr>
<tr>
<td>Basal limit of DEZ (µm)</td>
<td>248 ± 30</td>
<td>183 ± 76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Position of maximum</td>
<td>481 ± 50</td>
<td>417 ± 94&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>REGR (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal limit of CEZ (µm)</td>
<td>912 ± 137</td>
<td>1021 ± 108</td>
</tr>
</tbody>
</table>

<sup>a</sup>Difference in position is significant, P < 0.05
<sup>b</sup>P ≤ 0.10

**Table 2.1.** Comparison of growth profile features for vertical and gravistimulated *Arabidopsis* roots. The measurements are the axial distance from the root tip (mean ± SD).
in contrast to the response of the DEZ, the majority of the CEZ was inhibited on the upper side as well as the lower side. The basal region of the CEZ experienced the least change in growth rates; our estimates of the basal limit of the CEZ showed no significant changes after gravistimulation (P > 0.2).

Elongation of roots treated with an auxin-transport inhibitor. Since roots treated with auxin-transport inhibitors are agravitropic, we investigated the growth profiles of roots grown on plates containing 10 μM of the auxin-transport inhibitor NPA. Treatment with NPA reduced the growth rate from 175 ± 14 μm h⁻¹ (mean ± SE, n = 8) to 91 ± 7 μm h⁻¹ (n = 7). The extent of the elongation zone was reduced, so that the basal end of the CEZ shifted nearer to the root tip (Figure 2.6). This shift was caused by inhibition of growth in the CEZ. At the position of peak REGR in control roots, growth was inhibited by 25%, and 550 μm from the tip, the approximate midpoint of the CEZ, growth was inhibited by 81%. In contrast, growth in the DEZ was unaffected by treatment with NPA. The REGR at the DEZ-CEZ boundary (250 μm from the tip) was not significantly different from that of control roots (P > 0.6). Thus, NPA preferentially affects the CEZ.

Discussion

Because of the apparent importance of the DEZ in the response of roots to gravity and other environmental signals (Ishikawa and Evans 1993, 1995; Baluška et al. 1994), we were interested in characterizing the location of the DEZ in Arabidopsis roots. The DEZ has been defined based on cell shape changes in the apical part of the root (Baluška et al. 1990) and also on the basis of REGR profiles (Ishikawa and Evans 1993). Ishikawa and
Figure 2.6. Growth pattern for *Arabidopsis* roots treated with 10 µM NPA. The experiment was repeated 7-8 times. *Error bars* indicate SE.
Evans (1993) arbitrarily defined the basal limit of the DEZ of maize roots as the point along the REGR profile at which the rate of elongation reaches 30% of the peak rate in the central elongation zone. By this definition, in vertically growing roots of the Columbia ecotype of *Arabidopsis*, the basal limit of the DEZ is located approximately 250 μm from the root tip.

We had made an estimate of the basal limit of the DEZ in an earlier study, based upon patterns of change in cell shape, along with gravitropic behavior, at the root apex (Ishikawa and Evans 1997). The determination of the DEZ-CEZ boundary based upon cell shape patterns was about 150 μm farther from the tip than the location found in this study. However, cell shape patterns do not provide an adequate estimate of growth history in the absence of detailed information about cell division activity (Green 1976). This is of greater importance in *Arabidopsis*, because it has a steeper REGR profile in the apical region than maize (compare with Ishikawa and Evans 1991), resulting in a DEZ which is smaller relative to the CEZ, has a mean position closer to the meristem, and displays less total growth. This can explain the difference between the two estimates of DEZ location and the lower correlation between cell shape and DEZ position than for maize (see Figure 2.3). Also, Beemster and Baskin (1998) have found that the extent of the elongation zone can change with developmental age, suggesting that the location of the DEZ boundary may be dependent on root length. This age dependence also explains the larger elongation zone observed by Beemster and Baskin (1998).

Nevertheless, the location of the DEZ, as determined in this study, is consistent with recent data indicating that gravitropic curvature in roots of *Arabidopsis* is initiated in the DEZ (Ishikawa and Evans 1997). As shown in Figure 2.5, within 45 min of
gravistimulation the upper flank showed stimulation of growth in the DEZ, while growth on the lower side was inhibited in the DEZ. The maximum difference between the REGR of the upper and lower sides occurred 270 ± 50 μm from the tip, in close proximity to the basal end of the DEZ, with the magnitude of differential growth decreasing more quickly in the basipetal direction (data not shown, see Figure 2.5). This shows that a large proportion of the initial curvature originated in the DEZ. The relationship between root curvature and REGR profiles also depends on the difference in length of the upper and lower surfaces. In regions where bending occurred, the upper surface became longer than the lower surface. In these regions the lower side would require a higher REGR than the upper side just to maintain straight growth (Silk and Erickson 1978). Thus for these regions, namely the DEZ, the rate of curvature would be higher than the rate suggested by the difference in REGR alone.

In the CEZ, different changes occurred in the growth patterns following gravistimulation. Inhibition of growth on the upper side of the root in this region caused the position of peak REGR to shift acropetally. In the basal region of the CEZ, the REGR on the lower side of the root often increased, exceeding the REGR on the upper side of the root and causing upward curvature. This reversal in the growth rate gradient across the root is consistent with the finding in Lepidium of an area in the CEZ with reduced curvature during the gravitropic response (Selker and Sievers 1987). These multiple changes in the growth patterns of the root upon gravistimulation emphasize the complexity of the gravitropic response. On the other hand, the velocity at which a marker moved away from the tip at a particular axial distance was constant as a function of transverse position (Figure 2.4B). This means that any difference in growth rate
between the upper and lower flanks manifested itself simply as curvature, without an
axial transfer of growth through a slippage process. This confirms the assumption that
the location of curvature formation is the location of differential growth.

Because redistributions of the growth hormone auxin have been postulated to play
an integral role in gravitropism, inhibitors of auxin transport provide a tool for
investigating the relationships among auxin, growth, and gravitropism. NPA has been
shown to block polar auxin transport (Thomson and Leopold 1974, Katekar and Geissler
1980), as well as to inhibit gravitropism and root elongation (Gaither and Abeles 1975,
elongation and gravitropism has been suggested to be caused by the accumulation of
auxin to inhibitory concentrations in the root tip (Muday and Haworth 1994, Muday et al.
1995). Based on measurements of cell shape, Ruegger et al. (1997) suggested that this
growth inhibition is due to inhibition of the growth of cells nearest to the root tip
(including the DEZ). However, measurements of relative elemental growth rate (Figure
2.6) show the DEZ to be unaffected, with growth inhibition confined to the CEZ. This
pattern of growth inhibition may indicate the spatial distribution of NPA’s effect on auxin
transport. These results are also consistent with the model of auxin-independent
regulation of growth in the DEZ (Ishikawa and Evans 1993). However, since gravitropic
curvature is generated in the DEZ (Figure 2.5), it remains unclear how NPA inhibits
gravitropism.

This study shows the growth behavior of roots of *Arabidopsis* to be complex, with
fluctuations in growth patterns occurring across the whole length of the elongating
region, and differing for the DEZ and the central elongation zone. Such complex patterns
of growth are responsible for a variety of root growth behaviors including circumnutations (Johnsson and Heathcote 1973), root waving/coiling (Okada and Shimura 1990; Simmons et al. 1995b), and responses to environmental stimuli such as gravitropism (Zieschang and Sievers 1991), electrotropism (Ishikawa and Evans 1990b), and responses to chemical gradients (Ishikawa and Evans 1992). To better understand how these responses are coordinated, it will be necessary to determine the nature of localized changes in growth pattern and to identify the manner in which each tissue region contributes to the responses. Our characterization of the positioning of the DEZ and central elongation zone in Arabidopsis and the changes in growth which occur in these regions upon gravistimulation is an important step in assigning roles to these specific tissue regions in the response of roots to environmental stimuli.
CHAPTER 3

ROOT-GROWTH BEHAVIOR OF THE ARABIDOPSIS MUTANT rgr1: ROLES OF GRAVITROPISM AND CIRCUMNUTATION IN THE WAVING PHENOMENON

Although plant roots appear superficially to be symmetrical, their growth patterns can be asymmetrical. An example of asymmetrical growth patterns in a seemingly symmetrical environment is the skewed, wavy pattern observed in the roots of some ecotypes of Arabidopsis grown on inclined plates of agar (Okada and Shimura 1990, 1992; Simmons et al. 1995a; also see Figure 3.1A). The waving pattern is caused by a series of bends in the root that alternate in direction. The skew arises from a nonrandom preference by the root in the direction of bending relative to the vertical. There is a strict correlation between the direction of curvature for each bend of the root and the orientation (left or right) of the helical spirals formed by epidermal cell files, suggesting that the pattern is caused by a succession of left- and right-oriented processes (Rutherford and Masson 1996). The pattern of wavy growth has been suggested to result primarily from an interaction of gravitropism and thigmotropism (Okada and Shimura 1990, 1992) or from an interaction of gravitropism and circumnutation (Maher and Martindale 1980, Simmons et al. 1995b).
Figure 3.1. Waving/coiling patterns for seedlings of Ws (wild type, ecotype Wassilewskija) and rgr1. The roots were growing on the surface of agar (1% w/v) plates. A-C, Wild-type seedlings growing on plates tilted by angles of +30°, 0°, and -30°. D-F, rgr1 seedlings oriented at the same angles.
In the former model it is postulated that thigmotropism of the root tip causes a reversal in the direction of rotation of cell files and, therefore, a reversal in the direction of tip growth. Subsequent thigmostimulation of the root tip results in another reversal of cell-file rotation, causing the root to grow in a wavy pattern along the agar surface. Gravitropic sensitivity provides thigmostimulation by giving the root a tendency to grow into the agar.

According to the circumnutation/gravitropism model, the wavy pattern of root growth on inclined surfaces results from an endogenous pattern of root growth (circumnutation) interacting with the gravitropic response, which causes the roots to grow downward. Because root circumnutation usually has a chirality favoring the clockwise direction, as viewed looking along the root axis (Baillaud 1962, Johnsson 1997, and references therein), circumnutation may be able to provide the directional preference for the wavy growth. However, this slanting preference did not occur in roots embedded in agar, on a soft agar surface, or on a surface slanted so that gravitropism tended to pull the roots away from the agar surface (Rutherford and Masson 1996; also see Figure 3.1C). Therefore, asymmetrical mechanical stimulation, which these treatments minimize, is involved in the slanting response in some manner.

Because gravitropism serves an important role in these models of the waving pattern of growth, we wanted to investigate the relationships between this waving phenomenon and gravitropism and other growth behaviors in the gravitropism-deficient mutant rgr1 (reduced gravitropism of roots). The mutant was isolated from the DuPont T-DNA insertional mutagenesis collection in the Arabidopsis Wassilewskija ecotype by Simmons et al. (1995a) and is allelic to the axr4 mutant isolated by Hobbie and Estelle
Primary roots of these mutants are characterized by a slower rate of gravitropic curvature (Hobbie and Estelle 1995) and more random orientation about the vertical after gravitropic response (Simmons et al. 1995a). This confirms the importance of gravitropism in the waving phenomenon, because the roots of rgr1 seedlings grown on inclined surfaces form circular coils that have the same directional preference as the wild-type slanting preference, rather than forming wavy patterns (Simmons et al. 1995b, also see Figure 3.1D). In addition, the growth of roots of rgr1 seedlings is less inhibited by exogenously applied auxin than that of wild-type roots (Hobbie and Estelle 1995, Simmons et al. 1995a). This reduced sensitivity to auxin may explain the reduced gravitropism of rgr1 roots. If, according to the Cholodny-Went hypothesis, auxin redistribution between the upper and lower sides of the root following gravistimulation causes differential growth, then the reduced sensitivity to these auxin levels would lead to a reduction in differential growth in rgr1 roots.

We have developed an automated video-digitizer system for detailed measurement of the growth and curvature of roots of Arabidopsis. We used this new system to compare the kinetics of gravitropism and other growth behaviors between roots of rgr1 and wild-type seedlings in a series of different tactile environments. These comparisons provide insight into the basis of the waving/coiling growth phenomenon.

Materials and methods

Plant materials and growth conditions. Seeds of Arabidopsis thaliana (L.) Heynh of either the wild-type (ecotype Wassilewskija) or the mutant rgr1 were surface-sterilized
by agitation in a 5.25% (v/v) NaOCl solution for 5 min, followed by several rinses in distilled water. Seeds were sown in a row (three to five seeds per row) on sterile agar (1% w/v) in Petri dishes (60 mm diameter, 15 mm height) sealed with laboratory film (Parafilm, American Can, Greenwich, CT). The agar medium contained 1% (w/v) sucrose, \( \frac{1}{2} \) - strength Murashige-Skoog medium (Murashige and Skoog 1962), and 1 mM Mes, pH 5.8. The rows of seeds were placed on the agar surface perpendicular to the cylindrical axis of the dish so that the roots would grow along the surface or on a surface parallel to the axis, so that the roots would grow downward through the agar. The Petri dishes were either placed immediately in a culture room under continuous white light from fluorescent lamps (F30Y8-CW, Sylvania) with a fluence rate of approximately 50 \( \mu m \) m\(^{-2}\) s\(^{-1}\) at a temperature of 23°C, or they were refrigerated for 1 to 6 d before being transferred to the culture room. The Petri dishes were placed vertically or at the indicated angle of tilt and used for experimentation when the seedlings were 4 to 5 d old.

**Video-digitizer system.** The seedlings were viewed by a CCD (charge-coupled device) camera (Marshall Electronics, Culver City, CA) connected to a computer via a frame-grabber circuit board (ImageNation, Beaverton, OR). The roots were illuminated from behind with a fiber-optic illuminator (Fiber Lite, Leica) or with an IR light-emitting diode (Radio Shack, Fort Worth, TX). No difference in growth or response was observed between the two light conditions.

A new Windows-based software application was developed to analyze the growth behavior of roots in real-time, in terms of both the timing and extent of growth rate changes and localized curvature development, by combining a video data acquisition
process with an image analysis algorithm (Fig. 3.2). This software measures the rate of elongation of the opposite flanks of a root, as well as the orientation (relative to vertical) of different segments of the root, defined by their distance from the root tip. The software determines total elongation on opposite sides of the root by tracing the root edges from the calculated root-tip position (Figure 3.2A) to the position of fixed reference points in the non-elongating region every 20 to 60 seconds. Regression of the changes in length of a side with time gives its elongation rate. The software defines segments of the root by searching along arcs of fixed radius for the edges of the root (Figure 3.2B). A particular segment can then be described by a line segment connecting the midpoints of the arcs defining the boundaries of the segment. The angle of this line segment from the vertical is then calculated as a function of time.

*Interaction of gravitropism and tactile stimulation.* For the gravitropism experiments, root growth was first analyzed by the digitizer system with the seedling oriented vertically. The Petri dish containing the seedling was then rotated 90° in either a clockwise or a counterclockwise direction, and data collection was resumed. To determine the extent to which tactile stimulation of the root as a result of contact with the agar surface influenced the gravitropic response, gravitropism was measured both with the root growing along the surface of the agar and with the root growing through a solid block of agar. To minimize tactile stimulation, we also examined growth and gravitropism of roots growing in a liquid medium or in humid air. To create these conditions of minimal stimulation, we cut a small cube out of the agar block, just below the tip of the root. This created an open cavity, which was then covered with a layer of
Figure 3.2. Logic of custom software for measurement of length and orientation of roots. A key feature of the software is its ability to identify the edges of the root based on a user-defined threshold luminous intensity. A, Algorithm for identifying the root tip. 1) Based on a user-defined approximation or the previous root tip position (X), a circular arc is searched to find the edges of the root. From the edges, the midpoint of the arc is determined. 2) A line segment is traced from the midpoint of the arc toward the previous root tip position until the edge of the root is reached. One-quarter of the distance up this segment, a perpendicular is traced until the root edges are detected, allowing a new midpoint to be calculated. 3) The line connecting the arc midpoint and the new midpoint is extended to the edge of the root, which becomes the new root tip position (arrowhead). This process is repeated until it converges on a root tip position. B, Black and grey arcs denote segment limits as determined by the search routine of the software. The size of each segment is selected by the user and input as a searching radius. Black arcs indicate the limits of odd-numbered segments (1, 3, 5, etc.) and grey arcs indicate even-numbered segments. Segment angle is determined by first finding the arc connecting the edge points of the root at a given search distance, then determining the angle of the line connecting the midpoints of the two limiting arcs, relative to vertical. The overlapping process used to define segment limits increases the resolution of angle measurement. By definition the segments remain a fixed distance relative to the root tip. Total length of each edge is measured from the tip to a user-defined basal point in the mature region of the root (grey circles).
agar. For some experiments the cavity was filled with a liquid medium (½-strength Murashige-Skoog medium plus 1 mM Mes, pH 5.8).

*Circumnutation and calculation of curvature.* To observe the direction of circumnutation in a horizontal plane, root growth was measured in two perpendicular planes by placing two cameras at right angles to one another. The direction of curvature in the horizontal plane was then found by plotting the angle of the root tip in one plane in relation to the angle in the other. The curvature (the reciprocal of the radius of curvature) of points along this curve was calculated using seven-point quadratic numerical differentiation formulae, as described by Silk and Erickson (1978).

*Conventions of terminology.*

Angle of Plate Orientation. For purposes of this report, plates oriented vertically with the roots growing downward were assigned an angle of 0; plates tilted so the gravity vector pointed from the root toward the agar surface were assigned positive values of tilt; and plates inclined so that the gravity vector was away from the surface were assigned negative values.

Direction of Asymmetric Growth and Plate Rotation. For this report we assumed that the viewer’s perspective is looking at the seedling through the Petri dish cover with the seedling in front of the agar surface. From this perspective the directions of rotation are consistent with those in previous reports of root coiling (Mirza 1987, Simmons et al. 1995b). It is from this same frame of reference that we refer to the directional preference of the wavy growth pattern as slanting to the left.
Results

*Gravitropism and the waving response.* The wavy growth pattern of a wild-type root growing along an inclined plane has a directional preference to the left (Okada and Shimura 1990), which shifts the root's direction of growth away from the gravity vector; therefore, gravitropism should play a role in the wavy growth pattern. Indeed, the magnitudes of waving and of directional preference are dependent on the angle of incline (Figure 3.1, A-C). They are also angle dependent in *rgf1*, but the “waves” change to coils at positive angles (Figure 3.1, D-F). To investigate the interactions of gravitropism and waving/coiling, we analyzed the kinetics of gravitropism after seedlings were reoriented to a horizontal position with a clockwise or a counterclockwise rotation.

For roots of wild-type seedlings, the gravitropic responses after counterclockwise rotation (gravitropism and slanting are in the same direction; Figure 3.3A) and clockwise rotation (responses have opposing directional preferences; Figure 3.3B) were similar. In both cases, downward curvature was initiated in a region 200 to 300 μm from the root tip. It is in this region that roots show a characteristic divergence of orientation angle after gravistimulation (segments shown by arrows in Figure 3.3). Segments apical to this region show a change in angle attributable to the change of their basal neighbor, whereas segments basal to this region do not show curvature until growth causes the curvature to migrate back into these segments. Because we were able to localize the region of initial downward curvature, we calculated the time lag until curvature initiation as the time until the difference in angle between a segment apical to and a segment basal to the region of
Figure 3.3. Kinetics of the gravitropic response of a representative wild-type root growing along the surface of a vertically oriented agar plate. Arrows show the range of segments where the initial curvature originates. A, Root gravistimulated by a counterclockwise rotation to the horizontal. The length of the segments is 0.16 mm. B, Root gravistimulated by a clockwise rotation. The length of the segments is 0.20 mm. The stated segment distance is the distance from the root tip to the segment midpoint.
curvature reached 5% of the maximal difference minus the initial difference in angle. The difference in direction of gravistimulation did not cause a statistically significant difference in the time lag (Table 3.1), which averaged 27 min. As a measure of the magnitude of the growth response, we also calculated the average rate of curvature for the 2 h immediately following the time lag. The mean rate of curvature was 18° h⁻¹, with no significant difference between the clockwise and counterclockwise treatments (Table 3.1). Therefore, the directional preference of the waving was not strong enough to interfere with the wild-type gravitropic response.

The kinetics of the gravitropic response for the roots of rgrl, however, did depend on the direction of rotation (Figure 3.4). Seedlings rotated counterclockwise bent downward because of the initial curvature in the region 200 to 300 μm from the tip (Figure 3.4A). This response is similar to that of the wild type, except for the reduced rate of curvature (Table 3.1). However, for seedlings rotated clockwise, approximately 60% of the roots showed upward curvature (Figure 3.4B). This upward bending originates in the central elongation zone, farther back from the tip than the site of major downward curvature, which was found in the same location as in the wild type. Because of this upward curvature in roots rotated clockwise, there was a difference in the mean rates of curvature for the clockwise and counterclockwise treatments (Table 3.1). The time lags for the gravitropic response were unaffected in rgrl (Table 3.1).

The roots of rgrl showed a growth feature not found in the wild type: upward curvature in the central elongation zone. This feature has a clockwise directional preference, which is the same directionality found in the coiling response of rgrl. To
<table>
<thead>
<tr>
<th>Seedling type</th>
<th>Clockwise</th>
<th>Counterclockwise</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lag</td>
<td>Rate</td>
</tr>
<tr>
<td></td>
<td>min</td>
<td>min</td>
</tr>
<tr>
<td></td>
<td>h⁻¹</td>
<td>h⁻¹</td>
</tr>
<tr>
<td>Wild type</td>
<td>24 ± 6 (n = 6)</td>
<td>21 ± 7 (n = 6)</td>
</tr>
<tr>
<td>rgrl</td>
<td>25 ± 8 (n = 10)</td>
<td>2 ± 6ᵃ (n = 10)</td>
</tr>
</tbody>
</table>

ᵃ Significant difference (P < 0.05).

**Table 3.1.** Comparative gravitropism kinetics for roots of wild-type and rgrl seedlings growing on the surface of vertically oriented agar plates. Values are mean ± SD.
Figure 3.4. Kinetics of the gravitropic response of a representative root of an *rgr1* seedling growing along the surface of a vertically oriented agar plate.  

**A.** Root gravistimulated by a counterclockwise rotation to horizontal. *Arrow* indicates segments involved in initial downward curvature. Segment length is 0.10 mm.  

**B.** Root gravistimulated by a clockwise rotation. *Arrow* shows the upward curvature of the more basipetal segments, whereas the gravitropic response of more apical segments dampens this upward curvature. Segment length is 0.12 mm. Not all segments are shown.
determine whether the upward curvature was a true component of the gravitropic response or if it was part of the root-coiling pattern superimposed on the gravitropic response, we measured gravitropic kinetics under different tactile environments.

*Upward curvature and altered tactile and light environments.* To test the behavior of *rgrl* under a more uniform tactile environment, we measured the gravitropic response of roots growing through solid blocks of agar. Figure 3.5 shows the time course for the gravitropic response for such a root after clockwise reorientation. There was no initial upward curvature in the central elongation zone of roots growing through solid agar (*n* = 15). However, downward curvature occurred just as it did in roots growing along the surface (initiating 200 – 300 μm from the root tip). This suggests that an asymmetrical tactile environment is necessary for the upward-curvature component of the growth pattern. It is also consistent with the upward curvature being part of the root-coiling phenomenon, which also disappears when the root is embedded in agar (Rutherford and Masson 1996), rather than being a part of the gravitropic response.

To minimize the overall tactile stimulation, we also observed root behavior while the roots were growing in a liquid medium or in a chamber of humid air. The pattern of growth after clockwise stimulation was similar to that of roots growing in solid agar, with no upward curvature (E. Turk, unpublished data). To rule out light as a signal for upward curvature, we compared the behavior of a root growing along the agar surface after gravistimulation when the light source was in front of the plate and when it was behind it. We also measured the kinetics of gravitropism when the seedlings were exposed to no light during the experiment (except IR radiation for imaging by the camera). The upward
Figure 3.5. Kinetics of the gravitropic response of a representative root of *rgr1* growing through a block of agar. The root was gravistimulated by a clockwise rotation to a horizontal orientation. The experiment was repeated 15 times. Segment length is 0.10 mm. Not all segments are shown.
bending pattern does not depend on the type of light treatment provided (E. Turk, unpublished data).

_Circumnutation._ One model of the wavy growth phenomenon suggests that endogenous oscillations in the growth directions, called circumnutation, drive the pattern. Selective amplification of the pattern in an asymmetrical tactile environment could then create a directional preference. Therefore, we investigated roots growing vertically through a solid volume of agar for periodic oscillations in the root-tip direction. Roots of both wild type and _rg rl_ showed periodic oscillations in tip angle, with similar periods of 11.9 ± 1.1 h (mean ± SE; _n_ = 10) and 11.0 ± 1.7 h (_n_ = 5), respectively (Figure 3.6). Although the amplitude of the circumnutation seems variable, there was no obvious difference in the amplitude of the responses between _rg rl_ and wild-type seedlings. Therefore, the magnitude of the circumnutation was not dependent on the strength of the root's gravitropic response. There was no long-term directional preference evident in roots growing through agar.

This pattern of circumnutation in a uniform tactile environment (Figure 3.6) is very similar to the kinetics of the wavy growth pattern of a root growing along the agar surface (Figure 3.7). We measured the periodicity of this pattern as 11.1 ± 2.1 h (_n_ = 7) and 12.0 ± 2.2 h (_n_ = 5) for _rg rl_ and wild type, respectively, which is not significantly different from the circumnutation period. There was, however, a long-term directional preference for the root tip to stay to the left of vertical in roots growing along the surface, which can be seen by the shift in the angle of the axis of oscillations away from 0 (Figure 3.7).
Figure 3.6. Circumnutation patterns for roots growing through a block of agar. Oscillations in root tip orientation are shown for (A) a representative wild-type seedling and (B) a representative rgr1 seedling.
Figure 3.7. Waving growth pattern for roots growing along the surface of an agar plate. Oscillations in root tip orientation are shown for (A) a representative wild-type seedling and (B) a representative rgrl seedling.
To determine if the circumnutation behavior had any directional preference, we used two perpendicular cameras to measure the three-dimensional growth of rgrl and wild-type roots through solid agar (Figure 3.8). Although both clockwise and counterclockwise growth was observed, most root growth was in a counterclockwise direction, as viewed from above. If there were no directional preference to circumnutation, we would expect no mean curvature. However, the observed mean curvatures were $1.2 \pm 0.4$ milliradians$^{-1}$ (mean $\pm$ SE, $n = 10$) for rgrl and $1.8 \pm 0.7$ milliradians$^{-1}$ ($n = 7$) for wild type, significant differences from the theoretical mean ($P < 0.05$). Therefore, we found a chirality to circumnutation, even in a homogeneous tactile environment.

Discussion

These investigations of root behavior illustrate the complexities in growth kinetics that can occur as a result of the interactions of separate growth responses. Roots of rgrl seedlings tended to slant to the left when growing on vertical plates. This slanting contributed to the observed gravitropism kinetics in a manner dependent on the direction of rotation (Figure 3.4), suggesting that the relative magnitudes of the early curvature responses in rgrl may not have accurately reflected the difference in the strengths of the true gravitropic response. This was made more evident by the fact that the kinetics of the overall response of wild-type roots were not dependent on the direction of
Figure 3.8. A cross-sectional representation of changes in the root tip orientation of a typical wild-type seedling. The frame of reference is that of viewing the seedling from above. Relative times are given for the first and last observations.
Therefore, the magnitude of the gravitropic response of the wild type varied with the direction of rotation and offset the slanting preference of the root.

The interaction between slanting and gravitropism also illustrates the difficulty in calculating time lags based solely on changes in the angle of the root tip. Because we were able to localize the initial downward curvature to the region 200 to 300 μm from the tip, we could calculate time lags based on changes in angle occurring in this region only, excluding from consideration curvature arising farther back in the root, where the slanting phenomenon arises. The region initiating downward curvature corresponds to the location of the distal elongation zone (Mullen et al. 1998a, see Chapter 2), showing that rgrl roots have a functional DEZ. The upward curvature in gravistimulated rgrl roots occurred in the CEZ, which is consistent with the location of major bending in waving/coiling growth (Okada and Shimura 1990).

The lack of upward curvature after clockwise gravistimulation of rgrl roots growing in more uniform tactile environments (Figure 3.5) provides further evidence that the upward curvature is not a true component of gravitropism, but is a part of the wavy growth pattern. These results provide further evidence that an asymmetrical tactile environment is necessary for the establishment of a directional preference to the waving/coiling phenomenon.

The similarity in the frequencies of the wavy growth pattern and the circumnutation pattern of roots growing in uniform tactile environments (Figures 3.6 and 3.7) suggests that circumnutation may be the driving force behind the induction of tactile stimulation. Differences in growth rate also had no direct correlation with the period of the oscillations, which is in agreement with previous observations (Johnsson 1979).
frequency of waving remained the same despite differences in growth rate between roots growing on the surface of agar and those growing in the agar, or between rgr/ and wild-type roots growing in agar (J.L. Mullen and E. Turk, unpublished data).

An alternative to the model that circumnutation drives the waving pattern is the idea that a series of gravitropic and thigmotropic responses are sufficient to create the pattern. However, it seems unlikely that this scenario could explain the similarity in the frequency of the waving pattern in rgr/ and wild type, given their difference in gravitropic response. It is also difficult to explain the directional preference with such a model. Therefore, to account for all aspects of the waving/coiling phenomenon, both circumnutation and thigmostimulation are necessary components. In this combined model of the waving/coiling phenomenon, it is circumnutation that drives the pattern of wavy growth. Leftward slanting arose when the counterclockwise chirality of circumnutation caused differential thigmostimulation in an asymmetric tactile environment. Because of the chirality, the root was growing to the left at the point of greatest tactile stimulation. This thigmostimulation can manifest itself as an alteration in circumnutation amplitude. Further investigation into the nature of the three-dimensional circumnutation process may aid in the understanding of the tactile forces involved. The genetic analysis of mutants may also provide clues to the basis of the chirality. For example, sku mutants of Arabidopsis show increased magnitude of directional preference (Rutherford and Masson 1996), and Marinelli et al. (1997) found mutants with inverted chirality.

Whether the growth pattern takes the form of waving or coiling is based on whether the amount of bending in the clockwise direction during a half-wavelength of the
pattern is equal to the amount in a counterclockwise direction during the successive half-wavelength. For waving to occur, the sum of the gravitropic response and the thigmoresponse must be the same during clockwise and counterclockwise bending. Because the tactile stimulation is direction-dependent, the gravitropic response must be modulated to maintain a constant overall response, as seen for wild-type roots (Figure 3.3). The reduced gravitropic response of rgrl is unable to counter the differential thigmoresponse, so the amount of clockwise curvature is greater than the amount in the counterclockwise direction and the waving pattern switches to one of coiling.
Although the gravitropism of plant roots has been studied for well over 100 years, its mechanisms are still poorly understood. The use of Arabidopsis, with its plethora of mutants showing altered gravity response, promises to aid in the investigation of these mechanisms. Due to the small and fragile nature of the Arabidopsis root, characterizations of mutants are often performed with coarse temporal resolution, with the result that the initiation of the response is unobserved (Hobbie and Estelle 1995, Kiss et al. 1996, Fukaki et al. 1997, Tian and Reed 1999). Ishikawa and Evans (1997) and Mullen et al. (1998b) detailed the kinetics of gravitropic curvature in horizontally stimulated Arabidopsis roots. These studies revealed a time lag between 20 and 45 min. before curvature was initiated in the distal elongation zone (DEZ). Yet interpretation of data for later time periods is complicated by the process of the graviresponse, which changes the stimulation angle at the root cap, the probable site of gravity perception (for review, see Sack 1991). The response also creates a large difference in angle of orientation along the elongating region of the root and may involve adaptation to the gravity signal.
Sachs (1882) proposed that the gravitropic response was proportional to the component of the gravity vector perpendicular to the root axis, leading to the “sine rule” approximation of the dependence of response on the stimulation angle. While the rate of curvature has been found to be related to the sine of the stimulation angle for roots (Larsen 1969, Perbal 1974) and coleoptiles (Pickard 1973, lino et al. 1996), a simple sine dependence only held for angles of stimulation less than 90°, and the optimal angle of stimulation was in some cases greater than 90°. Further, a recent study of maize roots by Barlow et al. (1993) found no dependence of rate of curvature on stimulation angle, for angles between 20° and 90°, the range in which the sine rule approximation should be most valid.

Since prior studies of the relationship between stimulation angle and response in roots have been equivocal, we have employed a new technique to investigate this in the roots of Arabidopsis. We used a computer feedback system to rotate a seedling growing on agar so that its root tip was constrained to a particular angle from vertical. Thus we were able to maintain a constant stimulus throughout the graviresponse. This allowed us to evaluate the dependence of the response on stimulus angle and to better understand the detailed kinetics of the gravitropic response.

Materials and methods

Plant material and growth conditions. Seeds of Arabidopsis thaliana (L.) Heynh (ecotype Columbia) were surface-sterilized by agitation in 5.25% v/v NaOCl solution for 5 min., followed by several rinses in sterile distilled water. Seeds were sown in a row (3
seeds per row) on a sterile agar (1% w/v) medium in Petri dishes (60 mm diameter, 15 mm high) sealed with Parafilm (American Can Co., Greenwich, Conn., USA). The agar medium contained 1% w/v sucrose, ½-strength Murashige-Skoog medium (Murashige and Skoog 1962), and 1mM 2-(N-morpholino)ethanesulfonic acid (pH 5.8). The Petri dishes were either immediately placed vertically in a culture room under continuous white light from fluorescent lamps (Sylvania, Danvers, Mass., USA; F30T8-CW, fluence rate approx. 60 µm m⁻² s⁻¹) at 24 °C or refrigerated for 1-5 d before being transferred to the culture room. The seedlings were used for experimentation at age 4-5 d, when the roots had a length of 10-20 mm.

The feedback system. The seedling to be observed was first repositioned so that its root tip was at the center of the Petri dish (center of rotation). This minimized the translational movement of the root during the stimulation process. Repositioning was accomplished by placing forceps under the hypocotyl, lifting slightly, and sliding the plant along the agar. The dish containing the seedling was then attached to a vertical stage, and the seedling was allowed to recover for at least 1.5 h, during which time the growth rate of the root returned to normal (unpublished data, J.L. Mullen and C. Wolverton). The feedback system was then started, so that the root tip was constrained at the desired angle relative to vertical. The feedback system consisted of a CCD camera (Marshall Electronics, Culver City, CA, USA) focused on a seedling root, illuminated by an infrared light-emitting diode (Radio Shack, Fort Worth, TX, USA). The camera was connected to a computer via a frame grabber circuit board (Imageneration, Beaverton, OR, USA). A rotatable vertical stage (Optec Ltd., Tokyo, Japan) was also connected to the
computer and controlled by custom software. This software utilized the algorithm described by Mullen et al. (1998b; see Chapter 3) to calculate the angle of the root tip, as well as that of other root segments. The software constrained the user-defined segment of the root to the desired angle, making corrections as frequently as every 20 sec. Changes in the angle of the constrained segment caused a stepper motor to rotate the vertical stage in the necessary direction, correcting the angle of that segment (Figure 4.1). The custom software recorded the angle of the root segments and the rotation of the vertical stage. The root could be reoriented to a horizontal position (90°) within approximately 60 s by means of individual steps of the motor corresponding to 0.08°.

Results

Gravitropism kinetics. While experiments measuring the angle of a root tip as a function of time after gravistimulation provide useful measures of graviresponse, the strength of the stimulus is time-dependent in such cases. In order to better quantitate the kinetics of the graviresponse of Arabidopsis roots, we used the feedback system to smoothly reorient the roots from a vertical to a horizontal position. The tips of the roots were then constrained at an angle of 90° relative to vertical. The roots responded quickly to the gravitational stimulus and achieved a constant rate of curvature within an hour of stimulation (Figure 4.2). Curvature continued undiminished well beyond 90°, the amount of curvature that would be needed for the root tip to attain vertical orientation if it was not being constrained horizontally. Because the roots responded more quickly than we expected based on previous studies (Ishikawa and Evans 1997, Mullen et al. 1998b), we
Figure 4.1. Feedback system for constraining root orientation. A, After an image of the root is captured, the image is divided into segments, which are a fixed distance from the root tip. The angles of orientation for the segments are then determined and compared with the desired angle of constraint. The vertical stage is then rotated by the amount necessary to achieve the desired angle of orientation. B, Time-lapse images of an Arabidopsis root showing the development of gravitropic curvature while the root tip remains horizontal.
Figure 4.2. Kinetics of the gravitropic response of an *Arabidopsis* root. The root tip was constrained at 90° relative to vertical at 0 h. The measured rotation was the rotation of the vertical stage necessary to keep the root tip horizontal.
estimated the latent period for the response. To obtain an estimate of the latent period, we looked at the time interval of -0.5 to 1.5 h (Figure 4.3). A sixth-order polynomial was fit to data in this interval by nonlinear regression, and the second derivative with respect to time was taken. The local maximum of the resulting equation, which represents the time of greatest change in the rate of curvature, was used as the time of commencement of the response. Using this method, we calculated a latent period of $10.5 \pm 2.4$ min. (mean ± SE, $n = 12$). To better compare this estimate of the latent period with previous studies, we repeated the experiment, but we gravistimulated the root by removing the Petri dish from the vertical stage, manually rotating the root to a horizontal position, and reattaching the dish to the stage (Figure 4.3). The average latent period for manually stimulated roots was $19.6 \pm 2.6$ min. ($n = 9$), a significant difference ($P = 0.02$, t-test). Thus, it seems that the feedback system provided a reduction in mechanical stresses, which allowed a quicker response.

*Dependence on stimulation angle.* Because the curvature of the root as a function of time is linear over a long time interval (Figure 4.2), the average rate of curvature could be determined by linear regression. This value is a useful measure of the strength of the gravitropic response for a given angle of stimulation. To find the relation between the strength of the response and the dose of the gravity signal perpendicular to the root axis, we measured the mean rate of curvature for roots with tips constrained at different angles of stimulation. The rate of curvature increased with angle of stimulation for angles less than $90^\circ$ (Figure 4.4). The relation can be approximated by a sinusoidal function of the form:
Figure 4.3. Early time-course for the gravitropic response of typical *Arabidopsis* roots. The stage-rotated root was reoriented to a horizontal position by means of the rotating vertical stage. The manually-rotated root was removed from the stage, reoriented by hand, and reattached to the vertical stage. The fitted lines are sixth-order polynomials from which latent periods were calculated ($R^2 = 0.995$ and 0.976 for the stage-rotated and manually-rotated samples, respectively).
Figure 4.4. The dependence of the rate of root curvatures on the stimulation angle. Error bars indicate SE ($n = 10 - 12$). The curve is a sinusoidal function fitted to the data by nonlinear regression.
\[ y = a \sin(x + \phi) + y_0 \]

where \( y \) is the rate of curvature, \( x \) is the stimulation angle, and \( a, \phi, \) and \( y_0 \) are parameters calculated by nonlinear regression to be 15.9, -14.7, and 7.9 respectively. A physical interpretation of this equation is that the threshold angle for gravitropic response is 15°, since at this angle the sinusoidal term of the function is nil.

**Threshold angle for gravitropic response.** Since the previous data suggested that roots must be reoriented at least 15° from vertical before a response is elicited, we made measurements of gravitropic responses at low stimulation angles to test this prediction. Because the gravitropic response is characteristic in its rapid onset and constant rate of curvature (Figure 4.2), we were able to measure the proportion of roots, at a given angle of constraint, which responded to the gravitational stimulus. Figure 4.5 shows the proportion of roots responding as a function of stimulation angle. The angle at which 50% of the population responds gravitropically is approximately 15°, in good agreement with the prediction from the rate of curvature data.

**Localized contributions to the response.** Following gravistimulation, a root enters a phase of curvature development; but, after several hours, curvature in the elongating region, both within the apical region of the elongation zone (240 – 480 μm from the tip) and the middle of the central elongation zone (CEZ, 480 – 720 μm from the tip) reaches a steady state (Figure 4.6, segments 2 and 3 respectively). This steady state arises from a balance between continuing curvature formation and the migration of curvature out of the elongation zone as the root grows. A comparison of the deviation of steady state angles...
Figure 4.5. Threshold of gravitropic response in *Arabidopsis* roots. Roots were scored as responders if the root began curving within an hour of stimulation and continued curving throughout the course of the experiment ($n = 10$).
Figure 4.6. Localized changes in *Arabidopsis* root orientation upon gravistimulation. The data show the angles of orientation of different regions of a representative root when the tip-most segment is constrained at 120°. *Inset*, An image of the root, illustrating the division of the apical region into segments, numbered 1–3, corresponding to the angle data.
for different regions of the elongation zone from the angle of the root tip can be used as an index of relative contributions to the gravitropic response. Comparing the deviation of the apical region of the elongation zone to that of the middle of the CEZ (see Chapter 2, Mullen et al. 1998a for elongation zone analysis), we found that the apical region contributed the majority of the curvature when the root was reoriented to large angles (Figure 4.7). However, at smaller angles of stimulation, the more basal region of the elongation zone had significantly greater importance in the response than it had at larger stimulation angles (P = 0.01).

Discussion

By measuring the kinetics of Arabidopsis root gravitropism with a feedback system, we observed a rapid onset of curvature, averaging approximately 10 min. Therefore, the perceived stimulus must be transduced to a response in the DEZ and CEZ more rapidly than previously expected. The sedimentation time of amyloplasts has been measured to be approximately 5 min. (MacCleery and Kiss 1999). The presentation time, the minimum duration of stimulus required to elicit a gravitropic response, for Arabidopsis roots has been estimated to be between 20 s and 5 min. (Kiss et al. 1989, Kiss et al. 1996). It has previously been suggested that harsh gravistimulations may affect the sensing process (Barlow et al. 1993). While we did not attempt to measure presentation time using the rotating stage device, we expect that any changes in the presentation time will be reflected in the shorter latent period. Although potential electrical (Ishikawa and Evans 1990, Weisenseel et al. 1992) and chemical (Perera et al. 1999) signals have been
Figure 4.7. Relative local contributions to gravitropic curvature. The deviations in the orientation of the root tip from the central portion of the elongation zone and from the apical region of the elongation zone are compared to provide an index of the relative contributions of these regions to the gravitropic curvature. The ratio of these deviations was determined both for low angles of stimulation (n = 10) and high angles (n = 15). Error bars indicate SE. A large ratio indicates a relatively greater contribution to curvature by the central portion of the elongation zone, while a small ratio indicates a relatively greater contribution by the apical region of the elongation zone.
detected within this time interval, caution must be exercised when assessing components of the early phases of the gravitropic response. The manner in which roots were gravistimulated affected the observed latent period. Roots gravistimulated smoothly by the feedback system exhibited a reduced gravitropic latent period. This suggests that mechanical stress associated with rapid reorientation may alter the response and could confound the identification of true gravitropic signal transduction components.

Once the roots commenced bending, the rate of curvature remained constant over long time periods. This rate of curvature varied with the angle of stimulation and followed a sinusoidal dependence with a phase shift of 15°. This contrasts with observations in maize roots (Barlow et al. 1993), which found no relation between the rate of curvature and stimulation angle. These results also differ from older reports (see Audus 1964, Ferbal 1974) suggesting a "modified sine rule" with maximal curvature at angles of 120° to 135°. However, we were able to make observations at a constant root tip orientation for long periods of time. Since the root cap is the major site of perception of the gravity signal (Sack 1991, Blancaflor et al. 1998), allowing changes in the orientation of the root tip during gravitropic response can confound results. It seems likely that the large curvature observed at high angles of stimulation in other studies (Audus 1964) was caused by the root tip spending more time oriented at large angles rather than a larger magnitude response at these high angles.

The changes in the magnitude of the gravitropic response with stimulation angle were accompanied by changes in the location of the response (Figure 4.7). At small reorientation angles, much of the curvature occurred in the CEZ. However, as the stimulation angle increased, differential growth in the DEZ and the apical portion of the
CEZ increased more than in the central CEZ, shifting the region of greatest curvature acropetally. This type of response is consistent with a model of gravitropism in which the signal originates in the root cap and dampens with distance from the cap only if one assumes that the central CEZ is more sensitive to the signal than the more apical region of the elongation zone. To accommodate the observation that the region of maximal curvature shifts acropetally when the root is stimulated at higher angles (Figure 4.7), one may speculate that the apical region, because of its proximity to the signal source and the higher strength of the signal at higher stimulation angles, now receives a signal sufficiently above its response threshold to account for a large contribution to overall curvature. Alternatively, the apical region of the elongation zone might be capable of directly perceiving a gravistimulus for sufficiently large angles of stimulation, though this is a model which is difficult to reconcile with the observation that the gravitropic response is lost when the columella cells of the root cap are destroyed (Blancaflor et al. 1998).

This pattern of response is also consistent with the observed similarity between circumnutation and low-angle gravistimulation (Barlow et al. 1993) since circumnutation primarily occurs in the CEZ (Okada and Shimura 1990, Mullen et al. 1998b). Study of the growth patterns of roots constrained to angles below the gravitropic threshold may shed light on the relationship between circumnutation and gravitropism. The ability of the feedback system to smoothly rotate the seedling in order to maintain a constant angle of orientation for a specific region of the root, such as the root cap, provides a means for measuring curvature development while keeping the gravitropic stimulus constant. This
ability should prove useful in understanding the influence of gravity on other growth responses.
CHAPTER 5

CONCLUSIONS

We have developed an automated video digitizer system with custom image analysis software capable of measuring the growth responses of *Arabidopsis* roots with high resolution. By analyzing the displacement of markers applied to the root, we were able to characterize the relative elemental growth rate distribution along the elongation zone. The DEZ was found to extend back to 250 μm from the tip, while the CEZ extended to 900 μm from the tip (for roots between 10 mm and 20 mm in length). Peak growth occurred 480 μm from the tip. Inhibitors of transport of the growth hormone auxin altered this growth profile. Application of the transport inhibitor NPA specifically inhibited growth in the CEZ, leaving the DEZ unaffected. Since auxin transport inhibitors affect both growth and gravitropism, as well as the auxin-mediated process of lateral root formation, determining the location of NPA effects will help us understand the mechanism of its action. For example, these results cast doubt on the proposal that NPA inhibits gravitropism through non-auxin related inhibition of the DEZ (Evans and Ishikawa 1997, Ruegger et al. 1997). However, since a large proportion of differential growth during gravitropism occurs in the DEZ, it is unclear by what means gravitropism is inhibited.
We also utilized the digitizer system, without the application of markers, to investigate the interactions of gravity and other environmental stimuli. The gravitropic curvature was found to initiate primarily in the DEZ, consistent with the location of maximal differential growth. We also found a separate differential growth response in the gravitropic mutant *rgrl*. This response was localized to a different region of the root, the CEZ, and seems to be due to tactile stimulation in conjunction with circumnutation. The interaction of these differing responses is responsible for the waving/coiling phenomenon. Further work localizing responses to the different regions of the root may provide insights into the physiological differences between these two regions. For example, the DEZ and CEZ respond differently to applied electrical fields (C. Wolverton, J.L. Mullen, H. Ishikawa, M.L. Evans, unpublished data), which may indicate differences in electrical signaling.

Due to the high temporal resolution of the digitizer system, we were able to use the system to control a rotatable vertical stage, forming a feedback system. This allowed us to maintain root segments, such as the root cap, at constant orientations relative to vertical. Thus, the stimulus at the root cap remained constant throughout the experiments. In *Arabidopsis* the constant stimulus gave rise to a constant response. However, preliminary results in maize suggest a biphasic response (J.L. Mullen, C. Wolverton, unpublished data). Separating changes in the response due to changes in stimulus strength from those due to other changes such as signal transduction attenuation would advance our understanding of growth regulation. The control of root orientation, via the feedback system, will also be valuable in the study of responses to other environmental stimuli, by enabling us to maintain the root vertically, even as it responds.
to a stimulus. And by constraining the root at non-vertical orientations, we can study the interactions between gravity and other stimuli. Our findings also suggest that the process of gravistimulation can introduce mechanical perturbations, which may confound the interpretation of data regarding early signalling events. So care must be taken to minimize such additional stimuli.
APPENDIX

MEASUREMENT OF DIGITIZER SYSTEM ACCURACY

In order to make an empirical measurement of the digitizer system's accuracy in determining root orientation, we simulated roots of known orientation by generating root-shaped images. We created several test images with various orientations and input them into the digitizer system for analysis. We examined the effect of two variables on the accuracy and precision of the measurement of segment angle by the system: segment length and segment orientation. Although the variance decreased with segment length, in all cases, the mean angle measured by the system was within 0.5° of the true orientation, and the standard deviation of the measurements was less than 1° (Table A.1).

The accuracy of the system at calculating the root tip position was also determined by comparing the true root tip location of the test images with that calculated by the system. Since segment length affects the calculation of the root tip positions, we examined the effect of segment length on the error in root tip location. For all segment lengths tested, the mean error was less than 1 pixel, and the standard deviation of the measurements was also less than 1 pixel (Table A.2).
<table>
<thead>
<tr>
<th>Orientation (°)</th>
<th>30-Pixel Segment</th>
<th>40-Pixel Segment</th>
<th>50-Pixel Segment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.2 ± 0.6</td>
<td>-0.0 ± 0.3</td>
<td>0.1 ± 0.3</td>
</tr>
<tr>
<td>15</td>
<td>14.7 ± 0.6</td>
<td>15.2 ± 0.5</td>
<td>15.2 ± 0.3</td>
</tr>
<tr>
<td>30</td>
<td>30.1 ± 0.8</td>
<td>30.2 ± 0.5</td>
<td>30.0 ± 0.5</td>
</tr>
<tr>
<td>45</td>
<td>44.8 ± 0.8</td>
<td>45.1 ± 0.5</td>
<td>45.1 ± 0.4</td>
</tr>
<tr>
<td>60</td>
<td>60.2 ± 0.8</td>
<td>60.2 ± 0.7</td>
<td>60.2 ± 0.6</td>
</tr>
</tbody>
</table>

Table A.1. Accuracy of root orientation measurements. For all orientations and segment lengths, the measurement was repeated 30 times (stated values are mean ± SD).

<table>
<thead>
<tr>
<th>Segment Length (pixels)</th>
<th>Error in Root Tip Position (pixels)</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.67 ± 0.71</td>
</tr>
<tr>
<td>40</td>
<td>0.71 ± 0.52</td>
</tr>
<tr>
<td>50</td>
<td>0.80 ± 0.87</td>
</tr>
</tbody>
</table>

Table A.2. Accuracy of root tip position calculations. Measurements were repeated 30 times (stated values are mean ± SD).
LIST OF REFERENCES

Audus LJ (1964) Geotropism and the modified sine rule; an interpretation based on the amyloplast statolith theory. Physiol Plant 17: 737-745


Blancaflor EB, Fasano JM, Gilroy S (1998) Mapping the functional roles of cap cells


Dolk HE (1936) Geotropism and the growth substance. Rec trav bot néerl 33: 509-585


Firm RD, Digby J (1997) Solving the puzzle of gravitropism – has a lost piece been found? Planta 203: S159-S163


Navez AE (1933) Geogrowth reaction of roots of *Lupinus*. Bot Gaz 94: 616-618

Navez AE, Robinson TW (1933) Geotropic curvature of *Avena* coleoptiles. J gen Physiol 16: 133-145

Okada K, Shimura Y (1990) Reversible root tip rotation in *Arabidopsis* seedlings induced...
by obstacle-touching stimulus. Science 250: 274-276


mutant of *Arabidopsis thaliana* exhibiting altered auxin physiology. Physiol Plant 93: 790-798


